INFECTION AND IMMUNITY, Aug. 2005, p. 5256–5261 Vol. 73, No. 8
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.8.5256–5261.2005

Protection against Aerosolized *Yersinia pestis* Challenge following Homologous and Heterologous Prime-Boost with Recombinant Plague Antigens

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Received 14 January 2005/Returned for modification 15 February 2005/Accepted 16 March 2005

A *Yersinia pestis*-derived fusion protein (F1-V) has shown great promise as a protective antigen against aerosol challenge with *Y. pestis* in murine studies. In the current study, we examined different prime-boost regimens with F1-V and demonstrate that (i) boosting by a route other than the route used for the priming dose (heterologous boosting) protects mice as well as homologous boosting against aerosol challenge with *Y. pestis*, (ii) parenteral immunization is not required to protect mice against aerosolized plague challenge, (iii) the route of immunization and choice of adjuvant influence the magnitude of the antibody response as well as the immunoglobulin G1 (IgG1)/IgG2a ratio, and (iv) inclusion of an appropriate adjuvant is critical for nonparenteral immunization.

Recently, a great deal of attention has been directed towards needle-free immunization strategies as alternative methods for vaccine delivery. Both mucosal (intranasal [i.n.], oral, and rectal) and transcutaneous (t.c.) immunization in the presence of an appropriate adjuvant have been shown to induce humoral and cellular immune responses in both the systemic and mucosal compartments of immunized animals. Alternating routes for delivery of the priming dose and booster dose in immunizations, so-called prime-boost strategies, have also been examined. Such prime-boost strategies could be particularly important in an imminent or postrelease bioterrorism event if it is possible to administer a parenteral priming dose and, at the same time, distribute a follow-up patch, pill, or nasal applicator that could be self administered. Such vaccine strategies would greatly improve national preparedness.

In a recent study, we evaluated different prime-boost regimens, including parenteral, mucosal, and transcutaneous delivery, in order to explore the effect of changing the route of prime and boost on the ability of the recombinant *Yersinia pestis*-derived fusion protein (F1-V) to promote the development of long-lasting, high-titer antibodies (13). F1-V has been shown to provide protection against flea-borne, subcutaneous (s.c.), and aerosol challenge and has the potential to provide protective immunity against pneumonic as well as bubonic plague due to either wild-type $F1^+$ *Y. pestis* or to naturally occurring $F1^-$ variants (16, 17). The most significant finding of our previous study is that boosting by a different (heterologous) route than the priming dose can be as effective as or more effective than homologous boosting for induction of either serum or bronchoalveolar anti-F1-V immunoglobulin G1 (IgG1) responses.

In the current study, we examined the abilities of different prime-boost regimens with recombinant F1-V to protect mice against aerosol challenge with *Y. pestis*. We also examined the role of the coadministered adjuvant in inducing protection. For parenteral immunization, mice were immunized s.c. with 10 μg of F1-V alone or adsorbed to alum adjuvant (2.0% Alhydrogel, batch no. 3275; Superfos Biosector, Vedbaek, Denmark) brought to a final volume of 100 μl with 0.86 M NaCl. Mucosally and transcutaneously administered proteins are usually not immunogenic and also require the presence of an appropriate adjuvant. In the studies reported here, we utilized a mutant of the heat-labile enterotoxin of *Escherichia coli*, designated LT(R192G), that has been shown to be effective when administered mucosally (orally, rectally, or intranasally) or transcutaneously in a variety of animal models and in humans (2, 3, 5–7, 10, 12, 14, 19–24, 27–30, 32). Mice immunized i.n. received 5 μg of recombinant F1-V alone or admixed with 5 μg LT(R192G), brought to a final volume of 9.6 μl with TEAN (0.2 M NaCl, 0.05 M Tris, 0.001 M EDTA, 0.003 M NaN_3, pH 7.5), in one nostril following brief exposure to Isofluorane. Mice immunized t.c. received 35 μg F1-V alone or admixed with 25 μg LT(R192G), brought to a final volume of 50 μl with TEAN, applied to freshly shaved ventral skin following intraperitoneal injection of ketamine-xylazine. LT(R192G) was prepared in our laboratory by galactose-affinity chromatography as previously described (4). The vaccine antigen was a non-His-tagged version of the F1-V fusion protein, expressed by T7 polymerase with lactose operator control in *E. coli* strain BLR(DE3)/pPW731 and isolated to 99% purity with a four-column process (B.S. Powell, unpublished observation). Briefly, protein in clarified supernatant from disintegrated cells

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Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
1 AUG 2005	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Protection against aerosolized Yersinia pestis challenge following homologous and heterologous prime-boost with recombinant plague antigens, Infection and Immunity 73:5256 - 5261	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
Glynn, A Roy, CJ Powell, BS Adamovicz, JJ Freytag, LC Clements, JD	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

A Yersinia pestis-derived fusion protein (F1-V) has shown great promise as a protective antigen against aerosol challenge with Y. pestis in murine studies. In the current study, we examined different prime-boost regimens with F1-V and demonstrate that (i) boosting by a route other than the route used for the priming dose (heterologous boosting) protects mice as well as homologous boosting against aerosol challenge with Y. pestis, (ii) parenteral immunization is not required to protect mice against aerosolized plague challenge, (iii) the route of immunization and choice of adjuvant influence the magnitude of the antibody response as well as the immunoglobulin G1 (IgG1)/IgG2a ratio, and (iv) inclusion of an appropriate adjuvant is critical for nonparenteral immunization.

15. SUBJECT TERMS

Yersinia pestis, F1-V vaccine, vaccination route, adjuvant, laboratory animals, mice

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**SAR**	**6**	

TABLE 1. Immunization groups

Immunization groups[a]	Prime antigen	Prime adjuvant	Boost antigen	Boost adjuvant
Naïve				
i.n. × i.n.	5 μg F1-V		5 μg F1-V	
INr × INr	5 μg F1-V	5 μg LT(R192G)	5 μg F1-V	5 μg LT(R192G)
i.n. × s.c.	5 μg F1-V		10 μg F1-V	
INr × SCa	5 μg F1-V	5 μg LT(R192G)	10 μg F1-V	10 μl Alum
s.c. × s.c.	10 μg F1-V		10 μg F1-V	
SCa × SCa	10 μg F1-V	10 μl Alum	10 μg F1-V	10 μl Alum
s.c. × t.c.	10 μg F1-V		35 μg F1-V	
SCa × TCr	10 μg F1-V	10 μl Alum	35 μg F1-V	25 μg LT(R192G)
t.c. × s.c.	35 μg F1-V		10 μg F1-V	
TCr × SCa	35 μg F1-V	25 μg LT(R192G)	10 μg F1-V	10 μl Alum

[a] For parenteral immunization, F1-V was administered alone (s.c.) or adsorbed to alum (SCa). For mucosal and transcutaneous immunizations, F1-V was administered alone (i.n. or t.c.) or admixed with the mucosal adjuvant LT(R192G) (INr or TCr).

was denatured with 6 M urea at room temperature. F1-V protein was then captured and refolded by anion exchange chromatography, further purified and concentrated over tandem hydrophobic interaction chromatography columns, and exchanged into phosphate-buffered saline by size exclusion chromatography before flash freezing and storage at −80°C. Protein identity, quality, and structure were measured by several methods and determined to be as predicted. Bioburden in the form of nucleic acid and endotoxin ranged from 3 to 13 ng/mg and 25 to 379 endotoxin units/mg, respectively.

Survival of immunized mice following aerosol challenge with *Y. pestis*. As shown in Table 1, groups of 8- to 9-week-old female Swiss Webster mice were immunized twice (day 0 and day 28) with F1-V alone (s.c., i.n., or t.c) or adsorbed to alum (SCa) or admixed with LT(R192G) (INr or TCr), and groups of 10 animals from each regimen were challenged by aerosol with 70 50% lethal doses of *Y. pestis* (CO92) on day 87 following the primary immunizing dose of F1-V. The mice were challenged using a dynamic 30-liter humidity-controlled Plexiglas whole-body exposure chamber. Total flow through the chamber was 19.5 liters/minute and was maintained at atmospheric pressure throughout the exposure. The test atmosphere was continuously sampled by use of a 6-liter-per-minute all-glass impinger (Ace Glass, Vineland, NJ). Heart infusion broth with 0.001% (vol/wt) Antifoam A (Sigma, St. Louis, MO) was used as impingement collection medium. Nebulizer and all-glass impinger samples were plated after the exposure to establish the aerosol concentration within the exposure chamber. By use of the exposure concentration, an inhaled dose was estimated by multiplying the empirically determined aerosol exposure concentration (CFU/liter air) in the chamber by the amount of air that was estimated to have been breathed by the mouse during the exposure. The cumulative air breathed by each mouse during the exposures was calculated by estimating the respiratory minute volume based on Guyton's formula as previously described (15). For this study, the average challenge dose over four runs of the aerosol system, expressed in total inhaled CFU/mouse was 1.5×10^6 CFU. Survival was monitored for 216 h. Differences in survival between groups challenged with *Y. pestis* CO92 were analyzed by the Kaplan-Meier method with the log-rank Mantel-Haenszel test. Differences with *P* values of 0.05 or less were considered significant.

As seen in Fig. 1 and Table 2, all animals in the naïve control group succumbed to infection following aerosol challenge with *Y. pestis* with a median survival time (MST) of 72 h. By contrast, 9/10 positive-control animals immunized with an SCa prime and an SCa boost (SCa × SCa) with F1-V adsorbed to alum survived for the 216-h postchallenge observation period ($P < 0.0001$). Equivalent protection (9/10) was observed in animals primed INr and boosted INr in the presence of the adjuvant LT(R192G). Thus, homologous prime and boost with F1-V by either of the two routes in the presence of an appropriate adjuvant can provide significant protection against aerosol challenge. This is an important finding because it demonstrates that homologous mucosal immunization in the presence of an appropriate adjuvant can induce protection equivalent to parenteral immunization.

A primary objective of the experiments reported here was to determine if heterologous boosting could provide equivalent protection against aerosol challenge compared to homologous boosting. As shown in Fig. 1 and Table 2, there were no differences in the survival rates of groups of animals primed INr and boosted SCa (10/10), primed SCa and boosted TCr (9/10), or primed TCr and boosted SCa (10/10) (heterologous prime-boost) compared to animals primed SCa and boosted SCa (9/10) or primed INr and boosted INr (9/10) (homologous prime-boost) if an appropriate adjuvant was included in the immunization. Differences in survival were observed if animals were immunized with F1-V without an adjuvant, depending upon the route of immunization. Thus, animals primed s.c. and boosted either s.c. or t.c. without adjuvant in either the priming or booster dose had equivalent protection (s.c. × s.c. = 7/10; s.c. × t.c. = 8/10) that was not significantly different from the levels of protection observed by any combination of routes that included adjuvant. By contrast, animals that were primed nonparenterally (e.g., i.n. or t.c.) with F1-V without adjuvant and then boosted i.n. or s.c. without adjuvant had significantly lower survival rates (i.n. × i.n. = 0/10; t.c. × s.c. = 4/10; i.n. × s.c. = 3/10) compared to animals primed and boosted with F1-V in the presence of the appropriate adjuvant. As shown in Fig. 1 and Table 2, none of animals primed i.n. and boosted i.n. without adjuvant survived beyond 144 h postexposure (MST = 96 h), compared to 9/10 animals that survived for the duration of the experiment when primed INr and boosted INr with F1-V

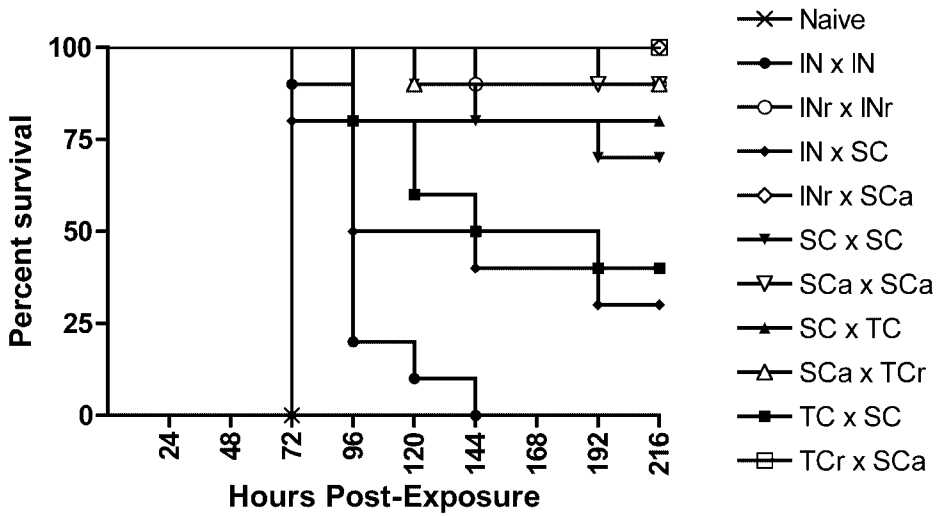

FIG. 1. Kaplan-Meier survival analysis of F1-V-immunized Swiss Webster mice after aerosol challenge with 70 50% lethal doses of *Y. pestis* (CO92) on day 87 postprimary immunization. There were no differences in survival rates of groups of animals primed INr and boosted SCa (10/10), primed SCa and boosted TCr (9/10), or primed TCr and boosted SCa (10/10) (heterologous prime-boost) compared to animals primed SCa and boosted SCa (9/10) or primed INr and boosted INr (9/10) (homologous prime-boost) if an appropriate adjuvant was included in the immunization. There were 10 mice per group.

admixed with the mucosal adjuvant LT(R192G) ($P < 0.0001$). Similarly, only 3/10 animals primed i.n. and boosted s.c. without adjuvant survived for the duration of the experiment (MST = 120 h) compared to 10/10 animals primed INr and boosted SCa with F1-V in the presence of adjuvant ($P = 0.0012$). Likewise, 4/10 animals primed t.c. and boosted s.c. without adjuvant survived for the duration of the experiment (MST = 168 h) compared to 10/10 animals primed TCr and boosted SCa with F1-V in the presence of adjuvant ($P = 0.004$).

Serum and bronchoalveolar lavage (BAL) anti-F1-V responses at the time of aerosol challenge following homologous or heterologous prime–boost. A cohort of mice immunized with F1-V adsorbed to alum (SCa) or admixed with LT(R192G) (INr or TCr) was sacrificed by CO_2 inhalation on the day corresponding to challenge (day 87 postprimary immunization) and their serum and BAL were examined for the presence of anti-F1-V, anti-F1, or anti-V antibodies by enzyme-linked immunosorbent assay (ELISA) on plates that were coated with 0.1 μg per well of recombinant F1-V, F1, or V in 100 μl bicarbonate buffer. Following overnight incubation at 4°C, plates were washed with phosphate-buffered saline containing 0.05% Tween 20, and twofold serial dilutions of the serum from immunized animals were applied. After incubation for 1 h at room temperature, plates were washed and a 1:400 dilution of goat anti-mouse IgG, IgG1, or IgG2a labeled with alkaline-phosphatase was added and incubation continued for 1 h at room temperature. Plates were washed, and the substrate paranitrophenyl phosphate was added. For quantitative analysis, concentrations of serum anti-F1-V, anti-F1, or anti-V IgG, IgG1, or IgG2a were determined by nonlinear regression from a standard curve of mouse myeloma IgG1 or IgG2a (Sigma Chemical Co., St. Louis, MO) serially diluted as a standard on each ELISA plate. The results obtained are expressed as the mean concentrations ± standard errors of the means (SEM). Statistical analyses were performed by using a one-way analysis of variance with Bonferroni's multiple comparison posttest. Statistical comparisons were performed with Prism version 4.0 (GraphPad Software Inc., San Diego, Calif.).

TABLE 2. Survival of immunized mice following *Y. pestis* aerosol challenge

Immunization groups[a]	% Survivors (216 h)	Median survival time (h)[b]
Naïve	0	72
i.n. × i.n.	0	96
INr × INr	90	N/A
i.n. × s.c.	30	120
INr × SCa	100	N/A
s.c. × s.c.	70	N/A
SCa × SCa	90	N/A
s.c. × t.c.	80	N/A
SCa × TCr	90	N/A
t.c. × s.c.	40	168
TCr × SCa	100	N/A

[a] For parenteral immunization, F1-V was administered alone (s.c.) or adsorbed to aluminum hydroxide (SCa). For mucosal and transcutaneous immunizations, F1-V was administered alone (i.n. or t.c.) or admixed with the mucosal adjuvant LT(R192G) (INr or TCr).

[b] Median survival time is the time at which 50% of the subjects have died. This value is not applicable (N/A) for groups with >50% survival.

Serum anti-F1-V IgG, IgG1, and IgG2a, as well as the serum anti-F1 and anti-V IgG responses in animals immunized with F1-V in the presence of an appropriate adjuvant, are shown in Table 3 and Fig. 2. Consistent with our previous findings, heterologous boosting was as effective as, or more effective than, homologous boosting for induction of significant anti-F1-V responses in immunized animals. The highest concentration of serum anti-F1-V IgG was obtained by heterologous prime-boost [INr prime with F1-V admixed with LT(R192G) and SCa boost with F1-V adsorbed to alum], and that was also reflected in the concentrations of anti-F1-V IgG1 and IgG2a (Table 3). With respect to serum anti-F1-V IgG1 and IgG2a

TABLE 3. Serum anti-F1-V (mean μg/ml ± standard error of the mean)

Immunization groups[a]	IgG	IgG1	IgG2a	IgG1/IgG2a ratio
Naïve	0	0	0	0
INr × INr	68 ± 27	14 ± 5	48 ± 31	0.3
INr × SCa	2,524 ± 1,427	443 ± 239	746 ± 495	0.6
SCa × SCa	374 ± 170	300 ± 201	53 ± 39	5.7
SCa × TCr	91 ± 40	46 ± 34	14 ± 5	3.3
TCr × SCa	58 ± 7	22 ± 4	9 ± 2	2.4

[a] Swiss Webster mice were primed INr, TCr, or SCa on day 0 and then boosted by the same route (homologous) or a different route (heterologous) on day 28. Animals were sacrificed on day 87 following the primary immunization. Blood was collected by cardiac puncture and analyzed by ELISA. The results obtained are expressed as the mean concentrations ± SEM. There were five mice per group.

ratios, animals that were primed INr had relatively lower IgG1/IgG2a ratios (INr × INr = 0.3; INr × SCa = 0.6) than did animals that were primed SCa or TCr (SCa × SCa = 5.7; SCa × TCr = 3.3; TCr × SCa = 2.4), with the most pronounced IgG1/IgG2a ratio resulting from SCa priming and SCa boosting with F1-V adsorbed to alum (Table 3). This shift in IgG1/IgG2a ratio could have resulted from either a route of immunization or adjuvant effect. With respect to BAL, all immunization groups that included adjuvant, regardless of route, developed significant levels of anti-F1-V IgG and IgG1. Animals that were primed INr and boosted SCa had the highest levels of overall BAL anti-F1-V IgG and anti-F1-V IgG1, and only those animals had detectable levels of BAL anti-F1-V IgG2a (data not shown). Additionally, BAL anti-F1-V IgA was not detected, and the concentration of BAL anti-F1-V IgG roughly corresponded to the level of serum anti-F1-V IgG, most likely indicating transudation of serum IgG into the BAL and not an active secretory process. Alternatively, the level of anti-F1-V BAL IgA may have been below the level of detection or may have peaked at a time point different than the sample time points in the experiments reported here. Serum anti-F1 IgG and anti-V IgG responses are shown in Fig. 2. Again, the highest concentration of either anti-F1 or anti-V was obtained by heterologous prime-boost [INr prime with F1-V admixed with LT(R192G) and SCa boost with F1-V adsorbed to alum]. Interestingly, there were no differences in protection against aerosol challenge between these immunization groups (Fig. 1 and Table 2).

The most significant findings of the study reported here are that (i) heterologous boosting protects mice as well as homologous boosting against aerosol challenge with *Y. pestis*, (ii) parenteral immunization is not required to protect mice against aerosolized plague challenge (i.n. × i.n. and s.c. × s.c. provide equivalent protection if an appropriate adjuvant is included in the vaccine formulation), (iii) the route of immunization and choice of adjuvant influence the magnitude of the antibody response as well as the IgG1/IgG2a ratio, and (iv) inclusion of an appropriate adjuvant is more critical for nonparenteral immunization.

The finding that a vaccine delivered by heterologous prime-boost can provide protection against aerosol challenge might have been predicted from our previous studies showing that the highest levels of anti-F1-V IgG1 were obtained by heterologous prime-boost. Related findings were reported by Eyles et al. (9), who demonstrated that t.c. application of F1 and V admixed with cholera toxin was effective for priming responses that could be boosted i.n. or intradermally and that t.c. application of F1 and V admixed with cholera toxin could effectively boost animals primed intradermally or i.n. However, the current study also demonstrates that i.n. priming in the context of an ADP-ribosylating adjuvant significantly lowers the serum IgG1/IgG2a ratio, indicating the development of more of a type 1 or mixed T-helper-cell response.

Moreover, INr × INr homologous prime-boost and SCa × TCr and TCr × SCa heterologous prime-boost all induced significantly lower levels of IgG1 than either SCa × SCa or INr × SCa immunization. Importantly, all of these groups had identical levels of protection against aerosol challenge. There are two possible explanations for the observed equivalent protection in the face of vastly different amounts of IgG1. First, there may be a threshold level of anti-F1 or anti-V IgG1 that is sufficient for protection and any of the combinations of routes in the context of an appropriate adjuvant can achieve that level. In that case, achieving the higher levels of antibody

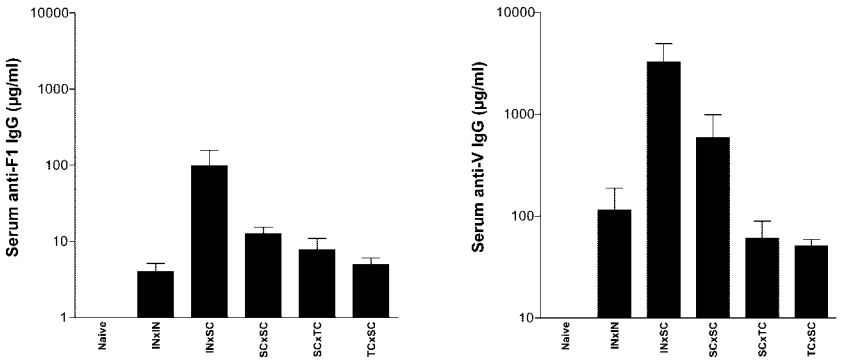

FIG. 2. Swiss Webster mice were primed INr, TCr, or SCa on day 0 and then boosted by the same route (homologous) or a different route (heterologous) on day 28. Animals were sacrificed on day 87 following the primary immunization. Blood was collected by cardiac puncture and analyzed by ELISA. Concentrations of serum anti-F1 or anti-V IgG were determined by nonlinear regression from a standard curve of mouse myeloma IgG1 serially diluted as a standard on each ELISA plate. The results obtained are expressed as the mean concentrations ± SEM. There were five mice per group.

would be important only if there was a concomitant increase in duration of circulating antibody or a relative increase in the challenge dose. The second possibility is that while anti-F1 or anti-V IgG1 may be correlated with protection, it may not be the sole protective factor. Indeed, a recent study by Elvin and Williamson (8) examined Stat6$^{-/-}$ and Stat4$^{-/-}$ mice to determine the relative importance of type 1 and type 2 immune responses in protection against plague challenge. Surprisingly, serum antibody responses to vaccination in both knockout strains were not different from wild-type controls with respect to levels of IgG or isotype profile. Moreover, Stat6$^{-/-}$ mice (unable to utilize type 2 cytokines interleukin 4 [IL-4] and IL-13) were highly protected against s.c. challenge, while Stat4$^{-/-}$ mice (inactivated IL-12 and interferon-γ-mediated immune mechanisms) were poorly protected, indicating that a type 1 immune mechanism, activated following Stat4 phosphorylation, may be essential for protection against plague. Thus, the undiminished protection following the observed shift to a type 1 or more mixed T-helper-cell response following i.n. priming in our study may reflect the contributions of both type 1 and type 2 responses to protection against aerosol challenge.

A number of studies have shown that the ADP-ribosylating enterotoxins can induce phenotypic and functional maturation of dendritic cells, as well as interacting directly with T-helper cells, B cells, and epithelial cells (1, 11, 18, 25, 26, 31). We did not include antigen-only (nonadjuvant) controls in the cohort immunization study, but future studies comparing adjuvanted and nonadjuvanted immunization groups could resolve whether the IgG1/IgG2a ratio shift is a function of the route of immunization or adjuvant.

The discovery that immunization by one route can prime for a secondary response by another route and protect animals against high-dose lethal aerosol challenge has far-reaching implications, especially for national preparedness in a biodefense or emerging infectious disease crisis.

These studies were supported in part by a grant from the U.S. Army Medical Research and Materiel Command.

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Editor: D. L. Burns